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Determination of 'Giardia' Cyst Viability

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16. ABSTRACT

The principal objective of this research was the development of a sensitive, standardized method of determining whether or not Giardia cysts are viable. The availability of such a method is necessary to determine the effect of chemical and physical agents on the viability of these organisms in water. Using cysts collected from an asymptomatic human carrier, a method for inducing excystation was developed and optimized with regard to pH, time of acid exposure, incubation temperature and excystation medium. The method was applied in determining the effects of cyst storage in water at various temperatures on cyst survival as determined by the cultural excystation method and eosin dye exclusion. The results indicate that cyst survival time decreased with increasing temperatures above 0°C. Freezing and thawing resulted in almost complete loss of viability.

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Determination of *Giardia* Cyst Viability

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July 1979

DETERMINATION OF *GIARDIA* CYST VIABILITY

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researchers and the user community.

This research provides a method for determining Giardia cyst viability which is apparently more sensitive than the previously-used dye exclusion methods. The development of this method makes possible its application in a variety of future studies, including:

1) the efficacy of presently-available water treatment methods in inactivating Giardia cysts,

2) the study of protozoan excystation, and

3) the establishment in culture of strains of Giardia trophozoites from cysts, rather than from sacrificed animals.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

ABSTRACT

The principal objective of this research was the development of a sensitive, standardized method of determining whether or not Giardia cysts are viable. The availability of such a method is necessary to determine the effect of chemical and physical agents on the viability of these organisms in water.

An asymptomatic human carrier of Giardia was found and provided the principal source of cysts for these experiments. A standard method of concentrating and purifying Giardia cysts from feces was developed.

A major contribution of this research has been developing a method of inducing, and determining the factors involved in, Giardia excystation. This method involves exposing cysts to pH 2.0 HCl for one hour at 37°C, and transferring the cysts to Giardia growth medium HSP-3 for one hour at 37°C. Variation of these conditions has revealed that the time of acid exposure, the incubation temperature and the composition of the post-acid incubation medium influence excystation levels.

Another contribution of this research has been the application of the excystation procedure as a method for assessing the viability of cysts. The effect of various storage temperatures on cyst survival in water has been examined. This study showed that survival time decreases with increasing temperatures above 0°C; near-freezing temperatures permitted longest survival. Freezing and thawing cysts resulted in an almost complete loss of viability.

This report was submitted in fulfillment of grant no. R 804898 by Ernest A. Meyer under the sponsorship of the U.S. Environmental Protection Agency. The report covers a period from October 20, 1976 to October 19, 1978.

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SECTION 1

INTRODUCTION

The problem addressed in the proposed study relates to the control of human infections caused by parasitic protozoa in the genus Giardia. Organisms in this genus are common intestinal inhabitants of man and a great variety of other animals. In various surveys in the last 30 years, the intestinal incidence of Giardia infection in humans has ranged from 1 to 10 percent.

Humans acquire Giardia infection by ingesting the cyst form of the organism; excystation takes place in the small intestine and the resultant trophozoites divide by binary fission and colonize the host. Trophozoites swept into the fecal stream lose their motility, round up, and are excreted as dormant, thick walled cysts which, upon being ingested by a suitable host, are capable of excysting and establishing another intestinal infection.

Giardia infections may be silent or symptomatic. Symptoms, when they occur, usually include a fatty diarrhea (steatorrhea) with epigastric pain and gas. Giardiasis may persist for years. The organisms apparently rarely invade the host's tissues. Children, dysgammaglobulinemic individuals and those with other immune deficits are particularly susceptible to symptomatic Giardia infections.

The epidemiology of giardiasis is similar to a number of other intestinal pathogens of man (viral, bacterial, and protozoal) in that the ultimate source of the organisms is (cyst-containing) fecal material and the portal of entry is the mouth. Drinking water containing Giardia cysts is believed to have been the vehicle for some of the giardiasis outbreaks in recent years, including outbreaks in Aspen, Colorado; Leningrad, Russia; and Rome, New York. The vehicle of a giardiasis outbreak in 1953-1954 in Portland, Oregon, which involved an estimated 50,000 cases, or one in Sydney, Australia, in 1975 involving hundreds of cases, has never been determined.

Inasmuch as public water supplies represent a potential vehicle for the spread of the cyst forms of these organisms, it is important that water be treated in a way that assures that no viable Giardia cysts are present. Because disinfection is often the means by which microorganisms are inactivated in water supplies, it would be of value to know whether Giardia cysts are in fact inactivated by usual methods of water treatment. Such a treatment method has not yet been obtained, because a reliable method of differentiating living and dead Giardia cysts is not available. The development of a method for determining cyst viability is an object of this research.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

This study has demonstrated that Giardia excystation, which normally occurs in the host gastrointestinal tract as a part of the infection process, can be regularly induced in vitro. Factors which affect excystation have been studied, and standard methods for concentrating and purifying cysts and inducing excystation have been developed. Preliminary comparison of eosin-exclusion and excystation as criteria of cyst viability suggests (1) that eosin-exclusion consistently indicates viability of cysts which are incapable of excystation, (2) that Giardia cysts are less hardy than previously indicated, and (3) that earlier criticisms of eosin-exclusion were well-founded. Excystation would seem to be the method of choice for determining Giardia cyst viability.

Excystation studies indicate that Giardia cysts survive well in near-freezing water; viability is reduced at higher temperatures. Freezing and thawing is detrimental to cyst survival.

Now that a sensitive, standardized excystation method is available for assessing Giardia cyst viability, it seems appropriate that it be used to obtain information that applies to the provision of safe drinking water, including:

(1) determining the effect of current chemical water treatment methods on Giardia cysts;

(2) determining the effect of other water treatments, including temperature, on Giardia cysts;

(3) identifying water treatment methods that will kill these organisms if present water treatment methods fail to inactivate Giardia cysts; and

(4) determining the relationship between excystation, eosin-exclusion and animal infectivity as indicators of cyst viability.

SECTION 3

MATERIALS AND METHODS

Cyst sources. Initial studies on excystation were carried out on cysts obtained from dogs (University of Oregon Health Sciences Center [UOHSC] Animal Care Facility, Portland, Oregon), from monkeys (Oregon Regional Primate Research Center, Portland, Oregon), and from hospital inpatients (UOHSC Department of Clinical Pathology). Some additional cyst-bearing animal feces were provided by other individuals and institutions. Later studies, and all those presented in this thesis, were performed using cysts obtained from one asymptomatic human male with giardiasis.

Cyst purification and storage. Cysts were purified by a modification of the procedures of Roberts-Thomson et al. (1) and Sheffield and Bjorvatn (2) as follows: Feces were suspended to a thin consistency in tap water and filtered successively through 1000, 710, 500, 250 and 177 µm-aperture nylon meshes. Three to 5 ml of filtrate was placed on 3 ml of chilled 0.85 M sucrose in a 15 ml conical centrifuge tube and the tube was centrifuged at 600 g for 5 minutes at room temperature in a swinging bucket rotor. The water-sucrose interface was removed, diluted 1:10 with water, and re-centrifuged for 5 minutes. The pellet was resuspended in 3 ml of water and the sucrose gradient centrifugation repeated as many times as needed to achieve the desired purity. Following this the water-sucrose interface was again removed, diluted 1:10 with water, filtered under vacuum through a 20 µm-aperture nylon mesh, and centrifuged for 5 minutes. This pellet was resuspended and diluted in tap water to a concentration of approximately 50,000 cysts/ml. In all experiments except those in which the effect of storage temperature was studied, cyst suspensions were stored at 8°C. The purification procedure could be accomplished in an hour yielding approximately 30 percent cyst recovery, with purity acceptable for light microscopic examination and enumeration, and apparently undiminished cyst viability.

Growth medium. *Giardia* growth medium HSP-3 derived from Meyer's HSP-1 and HSP-2 media (3) was used in the excystation and cultivation of trophozoites. This medium has the following formulation: 85 ml Hanks-phytone broth, 20 ml Seitz filter-sterilized heat-inactivated human serum, 7.5 ml NCTC-135 (Gibco), and 1.5 ml 1.0 M $NaHCO_3$; final pH 7.0. For culturing trophozoites, the following antibiotics were added to HSP-3: 250,000 units potassium penicillin G, 0.02 g streptomycin sulfate, and 0.01 g gentamicin sulfate.

Excystation solutions. Human saliva, gastric juice and duodenal-jejunal fluid were obtained from normal individuals (saliva) and from hospital inpatients (UOHSC Division of Gastroenterology Diagnostic Unit). The pH of

these solutions was measured on a Leeds and Northrup model 7401 pH meter and adjusted to desired pH values by the addition of dilute HCl or 1.0 M $NaHCO_3$.

Synthetic gastric juice, which was a composite of those described by Hirschowitz (4) and Konturek (5), contained major components of normal human gastric juice in aqueous solution as follows: $NaHCO_3$ (25 mN), KCl (12 mN), NaCl (40 mN), $CaCl_2$ (12 mN), HCl (120 mN), and pepsin (1500 units/ml; Sigma); final pH 1.6.

Aqueous HCl solutions were prepared by adding concentrated HCl to distilled water to the desired pH.

Excystation procedure. Unless stated otherwise the following excystation procedure was used in all excystation experiments: One volume of purified cyst preparation (usually 0.1 ml) was added to at least 10 volumes of excystation solution (saliva, gastric juice, HCl, etc.), and the mixture was incubated at 37 C for one hour. Following incubation, the suspension was centrifuged at 600 g for 5 minutes at room temperature, and the pellet resuspended in water and re-centrifuged. The pellet was then resuspended in HSP-3 (usually 0.5 ml) at 37 C. A depression slide chamber was filled with the suspension, sealed with a cover glass and paraffin-Vaseline, and incubated inverted at 37 C for one hour. The slide was then examined and the percentage of excystation determined. Timed experiments showed that a one hour incubation in HSP-3 was sufficient to allow complete escape of those trophozoites capable of excysting.

Quantitation of excystation and statistical analysis. Excystation was quantitated by systematic examination of slide chambers at 300x on a Unitron model PH-BMIC inverted microscope. Eight fields were examined on each cover glass in the pattern shown in Figure 1.

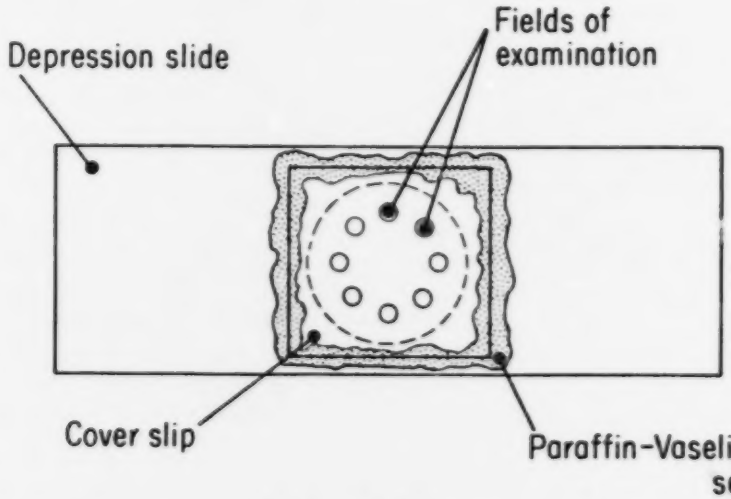

Figure 1. Pattern of the eight fields examined to determine percentage of excystation.

The fields were chosen such that they were mid-way between the outer edge and the center of the circular chamber, thus avoiding the sparsely populated outer edge and the densely populated center. This pattern was considered to be representative of the entire cover glass.

The percentage of excystation was determined by counting the number of intact cysts (IC), partially excysted trophozoites (PET), and totally excysted trophozoites (TET), and applying the following formula:

$$\text{Percent Excystation} = \frac{\text{TET}/2 + \text{PET}}{\text{TET}/2 + \text{PET} + \text{IC}} \times 100$$

In this formula the number of totally excysted trophozoites was divided by 2 because every cyst in which complete excystation occurred promptly yielded a pair of trophozoites. Totally excysting trophozoites rather than empty cysts were counted because: 1) empty cysts were sometimes difficult to detect due to their lack of refractility, 2) excysted trophozoites were easy to detect because of their active flagella, and yet seldom traveled appreciably, attaching to the glass almost immediately, and 3) the number of empty cysts present in a preparation due to causes other than excystation (e.g. death and trophozoite disintegration) were sometimes appreciable, particularly as the preparation aged. Trophozoite multiplication did not introduce additional counting errors since the generation time is approximately 18 hours [in M-5, a Giardia growth medium similar to HSP-3;(6)] Statistical significance was determined by Student's t-test.

Photomicrography. Photographs were taken under phase contrast at 630x on a Zeiss Invertoscope D with Kodak SO-115 film.

Effects of temperature on excystation. The effect of cyst storage temperature on excystation was examined by the following procedure: Purified cysts were suspended in water and stored at either -13°, 8°, 21° or 37°C; the effect of the latter three temperatures was studied in a single experiment using cysts purified from one fecal specimen. Cysts stored at -13°C were frozen in 0.5 ml aliquots by cooling to 8°C followed by transfer to -13°C; aliquots were thawed at 37°C as needed. At other temperatures, cysts were stored in 100 ml volumes from which aliquots were removed. Cyst viability was assessed periodically by excystation in HCl at pH 2.0 by the procedure described previously.

The effects of the acid and post-acid medium incubation temperatures were examined together. Cysts were exposed to pH 2.0 HCl at either 8°, 21° or 37°C by the usual procedure. The cysts exposed at one temperature were then divided into three equal portions (a total of nine for the three temperatures), and one portion from each temperature suspended and incubated in HSP-3 at either 8°, 21° or 37°C. These suspensions were observed after four and 18 hours incubation.

SECTION 4

RESULTS AND DISCUSSION

I. Induction and description of excystation.

Using the procedure described previously, excystation was induced in Giardia cysts and followed the course described below.

Excystation was seen minutes after the transfer to cysts into HSP-3, (Figure 2). The trophozoite first separated from cyst wall and began flexing movements, then began to emerge from the cyst. The emergence resembled the extrusion of a fluid-filled balloon through a small hole which was usually located in one end of the oval cyst. Active movement of flagella external to the cyst accompanied and possibly aided the escape of the trophozoite. Cell division always followed the emergence of a trophozoite from the cyst. Often the process of differentiation and division was nearly complete even before the trophozoite was free from the cyst wall. The entire process of excystation required from five to 30 minutes following transfer of cysts into HSP-3.

Abortive excystation was noted in three forms. In the first, the flexing movement inside the cyst was followed by a cessation of trophozoite activity and a "re-expansion" of the trophozoite to fill the cyst. In observations of cysts of this type over several hours, no subsequent activity was seen, indicating loss of viability. In the second form of abortive excystation the trophozoites appeared to have died while attempting to escape from the cyst, resulting in a final stage which appeared like one of those in Figure 2B-D; in this stage cytoplasm of the intra- and extra-cystic portions of these partially excysted trophozoites appeared structurally normal. In the third form, cysts appeared similar to the one in Figure 2B, except that the protruding cytoplasm was devoid of any discernible internal structure or organelles. In addition, the number and size of the protrusions varied; when multiple protrusions were present they were always localized in one area of the cyst surface. Protrusions apparently "budded-off" completely from some cysts and could be seen as refractile spheres free in the medium. The frequency of cysts exhibiting abortive excystation seemed to increase with increasing cyst age.

Later experiments revealed that aberrant, as well as abortive, excystation could occur. Occasionally trophozoites attempted excystation through the side of the cyst (lateral excystation) rather than through one end (terminal excystation). In these, excystation was almost always abortive. Unusual extra-cystic structures were rarely observed. These

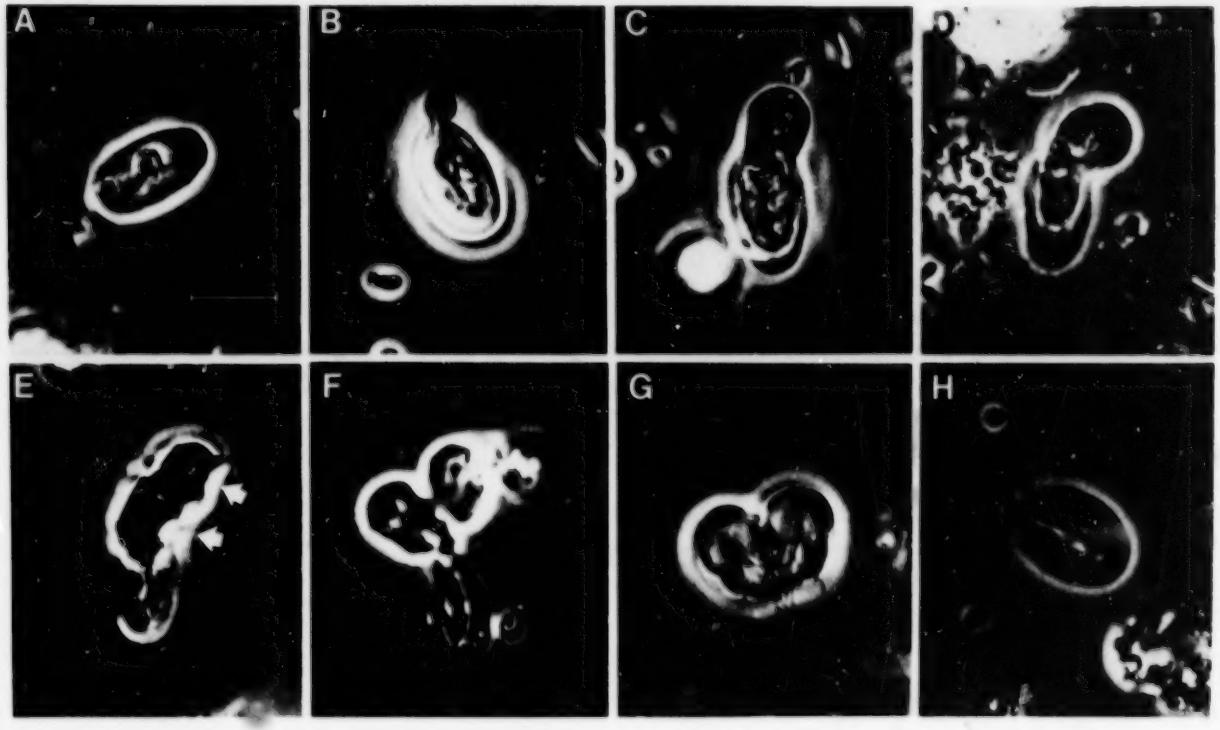

Figure 2. Representative steps in Giardia excystation. (A) Typical cyst; (B through F) sequential emergence of trophozoite(s) [arrows (E) indicate ventral adhesive discs of daughter trophozoites]; (G) excystation completed, division of daughter trophozoites continuing; (H) empty cyst. Scale bar = 10 μm.

included motile trophozoite-like organisms approximately ¼ to ½ normal trophozoite size, and rod-like or club-shaped structures resembling axostyles surrounded by cytoplasmic remnants "wiggling" independently in the medium. The incidence of aberrant excystation and of unusual extra-cystic structures was greater in some fecal specimens than in others -- even from the same host. In one human case followed from onset of symptoms through eight months of asymptomatic infection, most fecal specimens contained typical cysts yielding up to 60 percent excystation and normal, active trophozoites. Other fecal specimens contained cysts which appeared typical, but in which the ability to excyst was either poor or absent. Still other specimens yielded irregularly shaped cysts containing either normal or abnormal-appearing trophozoites. These atypical cysts were likely to undergo aberrant and/or abortive excystation, and were found with increasing frequency in fecal specimens as the patient approached a three-month period of non-excretion.

II. Identification of inducing factor.

In an attempt to duplicate the conditions to which cysts are exposed in vivo, purified Giardia cysts were exposed to human upper alimentary tract fluids according to the excystation procedure described previously. Due to a shortage of fluids, only one trial was performed, and approximately 100 cysts were counted at each pH in each fluid. It was found that only those fluids with a pH of 5.0 or below induced excystation and that the percentage of excystation appeared to increase with decreasing pH (Table 1).

TABLE 1. EXCYSTATION OF GIARDIA EXPOSED TO HUMAN UPPER ALIMENTARY TRACT FLUIDS

Fluid	pH	Percent Excystation
Water (control)	6.8	0
HSP-3 (control)	6.8	0
Saliva	7.6	0
Gastric juice*	7.6	0
Gastric juice	5.0	10.9
Gastric juice#	2.4	40.8
Duodenal-jejunal fluid	5.7	0
Duodenal-jejunal fluid	4.3	10.6

* From gastric resection patient (patient 1).

From patient 1, adjusted to pH 2.4.

The influence of pH on excystation was examined by adjusting specimens of human saliva, gastric juice, and duodenal-jejunal fluid to various pH values and inducing excystation in these fluids. Again, one trial was performed and approximately 100 cysts were counted at each pH level, in each fluid. The results of this experiment are shown in Table 2.

TABLE 2. EXCYSTATION OF GIARDIA EXPOSED TO pH-ADJUSTED HUMAN UPPER ALIMENTARY TRACT FLUIDS

Fluid	pH	Percent Excystation
Water (control)	6.8	0
HSP-3 (control)	6.8	0
Saliva	7.6	0
	2.3	43.8
Gastric juice	7.6	0
	6.8	0
	5.9	0
	4.9	18.0
	4.0	42.5
	2.9	30.3
	2.1	37.2
Duodenal-jejunal fluid	7.2	3.1
	2.3	48.6

All specimens with pH values between 2.1 and 4.9 induced high levels of excystation; little or no excystation was induced at higher pH values. In gastric juice the highest levels of excystation were induced between pH 2.1 and 4.0, with reduced excystation at pH 4.9 and none at higher pH values.

A synthetic gastric juice was prepared and used in an attempt to identify the factor(s) inducing excystation in normal gastric juice. Aliquots of the synthetic fluid were adjusted to different pH values and excystation performed in these solutions. Eight trials were performed in which an average total of 1825 cysts were counted at each pH. The results (Figure 3) were similar to those found in experiments using human gastric juice. Peak percentages of excystation were observed between pH 2.0 and 3.2 with progressively diminishing levels above or below these pH values. Peak percentages of excystation were significantly greater than those at other pH values ($p<0.05$).

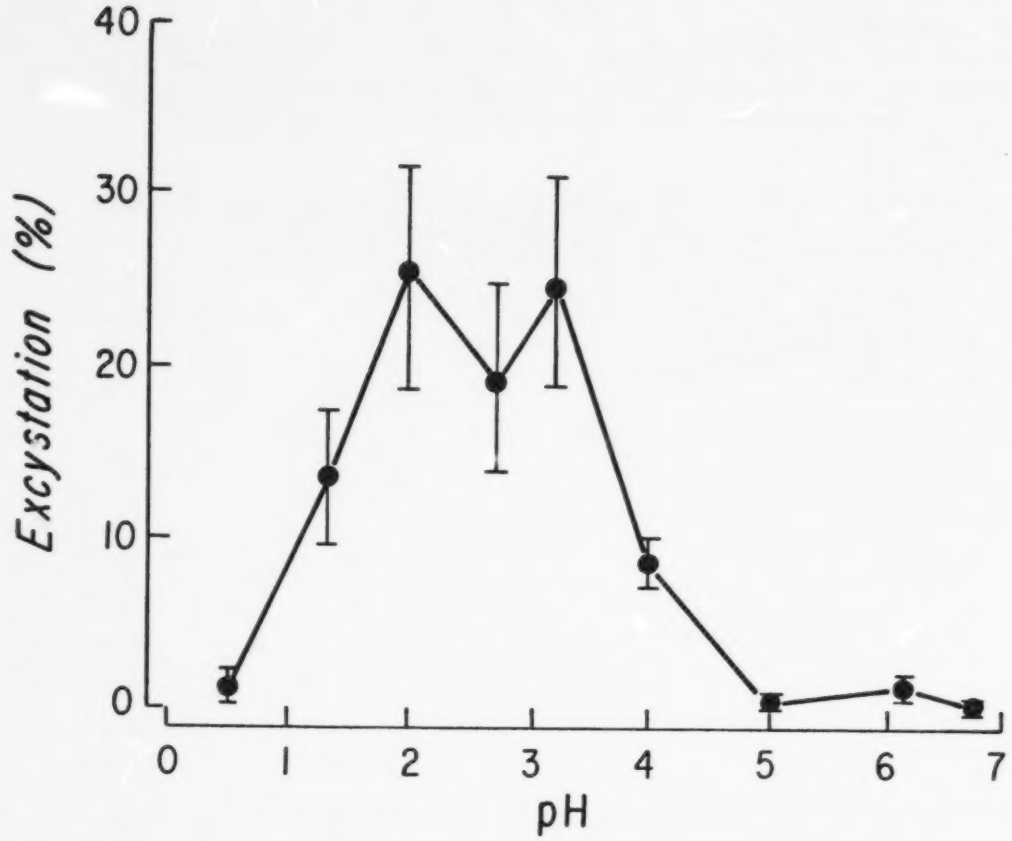

Figure 3. Excystation of Giardia exposed to pH-varied synthetic gastric juice. Vertical bars represent standard error of the mean.

To identify factors other than pH influencing excystation, complete and component-varied synthetic gastric juices were prepared and compared for ability to induce excystation. Eight separate trials were performed in which an average total of 1459 cysts were counted in each solution. With the exception of water and HSP-3 controls, all of the solutions induced almost identical levels of excystation ($p>0.05$; Table 3); excystation in the control tubes was significantly lower than in the other solutions ($p<0.05$). The only common factor in the excystation-inducing solutions was HCl in a concentration resulting in a pH of 1.6. The presence of salts and pepsin did not significantly alter the degree of excystation.

TABLE 3. EXCYSTATION OF GIARDIA EXPOSED TO COMPLETE AND COMPONENT-VARIED SYNTHETIC GASTRIC JUICE

Solution	pH	Mean * percent excystation ± S.E.M.
Compete (HCl + salts + pepsin)	1.6	25.8 ± 7.3
HCl + salts	1.6	23.0 ± 7.0
HCl only	1.6	23.2 ± 7.0
Water (control)	6.8	0.1 ± 0.1
HSP-3 (control)	6.8	0.0 ± 0.0

* Means are derived from an average total of 1459 cysts counted in each solution.

To examine the influence of HCl on excystation, aqueous HCl solutions at various pH values were prepared and eight excystation trials were performed in each solution; an average total of 1564 cysts were counted at each pH. The results (Figure 4) revealed a pattern similar to those observed when pH-varied gastric juice and synthetic gastric juice were used.

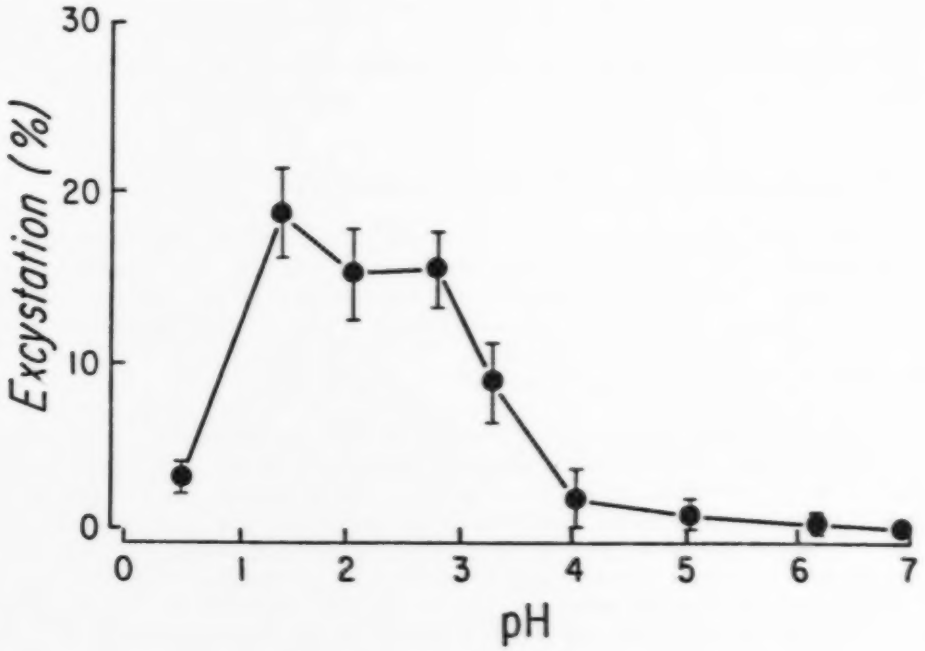

Figure 4. Excystation of Giardia exposed to pH-varied HCl. Vertical bars represent standard error of the mean.

The percentages of excystation at pH 1.3 to 2.7, although not significantly different from each other ($p>0.05$) were significantly greater than those at other pH values ($p<0.05$).

To determine whether the factor inducing excystation was hydrogen ion, chloride ion, or a combination of the two, several mono-, di-, and trivalent inorganic acids were diluted in water to a final pH of 2.0 and excystation attempted. Eleven trials were performed, an average total of 2242 cysts counted in each acid. Each acid induced almost identical levels of excystation ($p>0.05$; Table 4).

TABLE 4. EXCYSTATION OF GIARDIA EXPOSED TO INORGANIC ACIDS AT pH 2.0

Acid	Mean * percent excystation ± S.E.M.
Water (control)	0.2 ± 0.2
HCl	17.4 ± 2.2
HNO_3	12.5 ± 3.0
$HClO_4$	15.5 ± 2.0
H_2SO_4	15.5 ± 2.6
H_3PO_4	19.4 ± 2.8

* Means are derived from an average of 2242 cysts counted in each solution.

III. Effect of variation of physical environment.

A. Hydrogen and other ions. As shown previously, the induction and level of excystation is dependent upon pH (Tables 1 and 2, Figures 3 and 4). Neutral or near-neutral-pH solutions failed to induce excystation regardless of their complexity (Tables 1 and 2, Figures 3 and 4), with exceptions to be discussed later. Other than hydrogen, the ions tested did not appear to influence the degree of excystation (Tables 3 and 4).

B. Time of acid exposure. To examine the influence of acid exposure time on excystation, cysts were exposed to HCl at various pH values for intervals from five minutes to four hours. Five trials were performed counting an average total of 1449 cysts at each pH-time combination. The results, shown in Table 5, indicated that: 1) the exposure time required for excystation is reduced as pH decreases; 2) an optimum range of exposure times exists at pH values from 0.5 to 6.2, and at any one time an optimum pH interval can be determined; and 3) the mean optimum exposure time within this range increases as the pH increases. Additionally, the mean optimum percentage of excystation at pH 0.5 and 2.0 were not significantly different from each other, yet were significantly greater than those at 4.0 and 6.2 ($p<0.05$).

TABLE 5. EXCYSTATION OF GIARDIA BY VARYING pH AND EXPOSURE TIME

pH	Exposure time (minutes)										Mean optimum# exposure time	Mean optimum percent excystation@
	5	10	15	20	30	60	90	120	180	240		
0.5	243*	256	231	--**	127	38	--	--	--	--	10	243
2.0	28	248	--	222	203	100	53	20	--	--	20	224
4.0	0	1	--	5	28	23	2	9	2	--	45	26
6.2	--	--	--	--	0	3	8	6	4	0	120	5

* Values represent percentage of excystation with reference to control tube (pH 2.0, 60 minutes) which was designated arbitrarily as 100 percent. Underlined values represent optimum percentages of excystation at a given pH which are not significantly different from each other ($p>0.05$).

** Excystation was not determined at these combinations.

Average of exposure times at which optimum percentages of excystation were not significantly different from each other at a given pH ($p>0.05$).

@ Average of optimum percentages of excystation which are not significantly different from each other at a given pH ($p>0.05$).

C. Temperature. The influence of temperature on cysts and excystation was examined in three aspects: 1) the effect of storage temperature, 2) the effect of the temperature of the acid incubation solution, and 3) the effect of the temperature of the post-acid incubation medium (HSP-3).

The effect of storage at -13°, 8°, 21° and 37°C on cyst viability was assessed periodically as described previously. An overall average of 440 cysts were counted at each temperature on each day tested. Representative results of these experiments are summarized in Figures 5-8. Cysts stored at 8°C (Figure 5) had higher levels of excystation for a longer duration than those stored at the other temperatures. The maximum percentages of excystation observed at each storage temperature were in the order 8°>21°> 37°>-13°C. Viability at 8°C was observed through day 77 at which time the cyst suspension was depleted. At 21°C (Figure 6), cyst survival ranged from 5 to 24 days. Cysts stored at 37°C (Figure 7) never survived longer than four days. Usually the percentage of excystation was greatly reduced, even after 24 hours, as indicated by an experiment in which excystation was monitored hourly, counting an average of 117 cysts at each temperature for each hour. In this experiment (Figure 9) the percentage of excystation for cysts stored ranged from 40 to 60 percent over the 24-hour period; at 37°C, following initial peaks of excystation, the percentage of excystation declined to less than 10 percent after 7 hours, and diminished gradually over the next 18 hours. At -13°C storage (Figure 8) the cysts showed almost a total loss of viability within 24 hours. Only 0.2% of the cysts survived to day 11 at this temperature.

An interesting feature of storage at 8° or 21°C was that in all cyst suspensions obtained from fresh fecal specimens, a period of low excystation, varying from two to seven days and followed by an increase in excystation, was observed.

The effects of the HCl and HSP-3 incubation temperatures were examined together as described previously. Five trials were performed, in which an average total of 379 cysts were counted at each temperature combination. The results of this experiment (Table 6) showed that excystation is dependent upon both the acid and the HSP-3 incubation temperatures. The highest

TABLE 6. EXCYSTATION OF GIARDIA BY VARYING THE TEMPERATURE OF HCL (pH 2.0) AND HSP-3

Acid temperature (°C)	HSP-3 temperature* (°C)		
	8	21	37
8	0	0.5	1.5
21	0	3.0	5.4
37	0	15.6	100.0

* Values represent percentage of excystation with reference to the control tube (HCl 37°C, HSP-3 37°C) which is arbitrarily 100 percent.

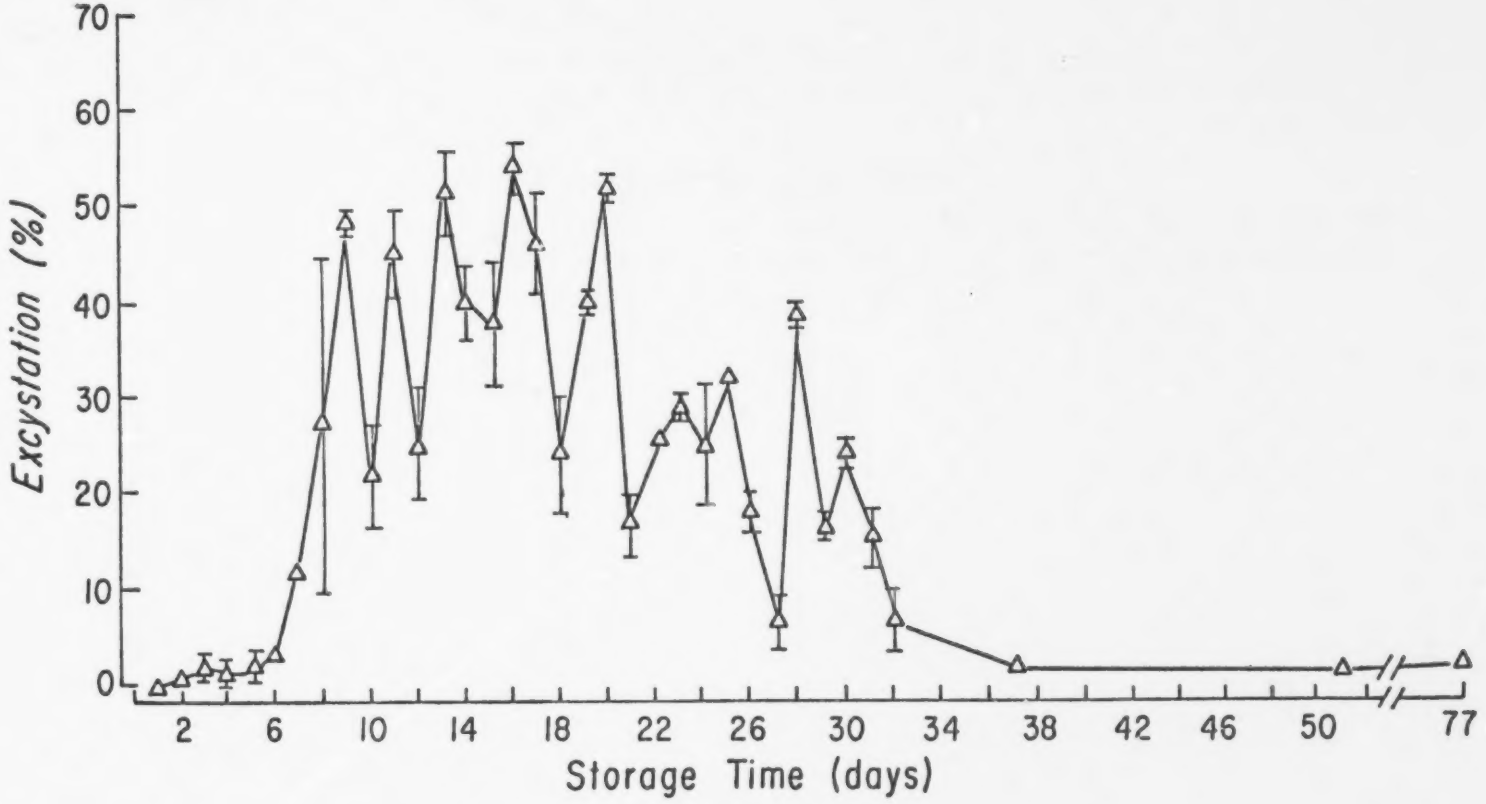

Figure 5. Effect of storage at 8°C on Giardia cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

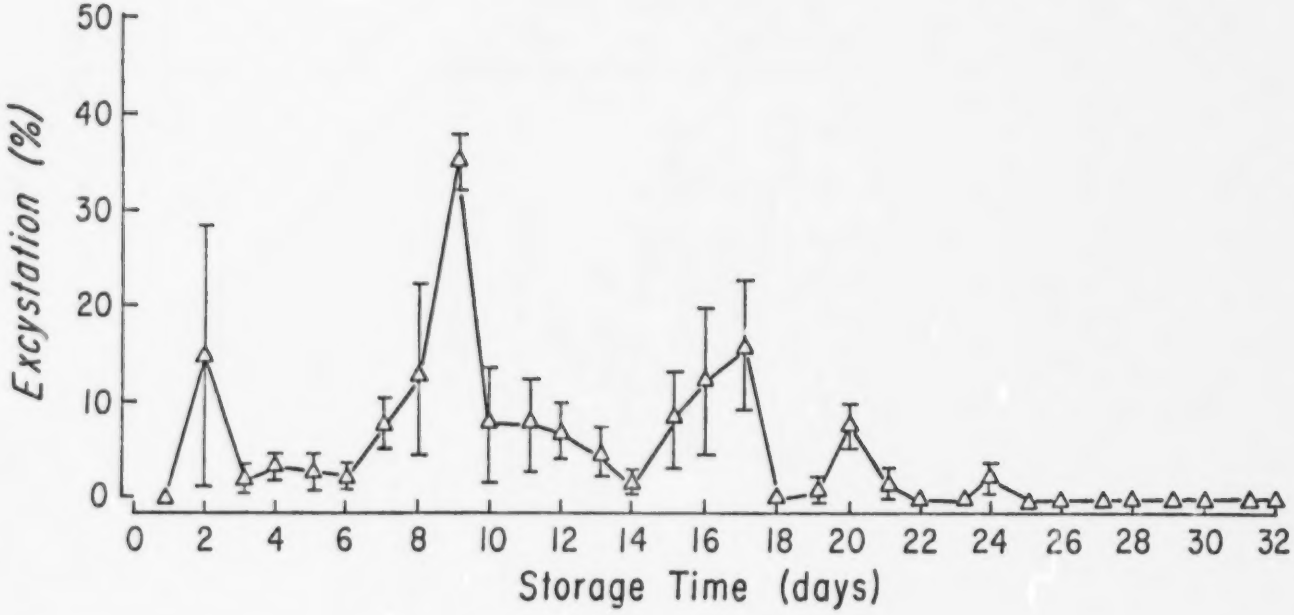

Figure 6. Effect of storage at 21°C on Giardia cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

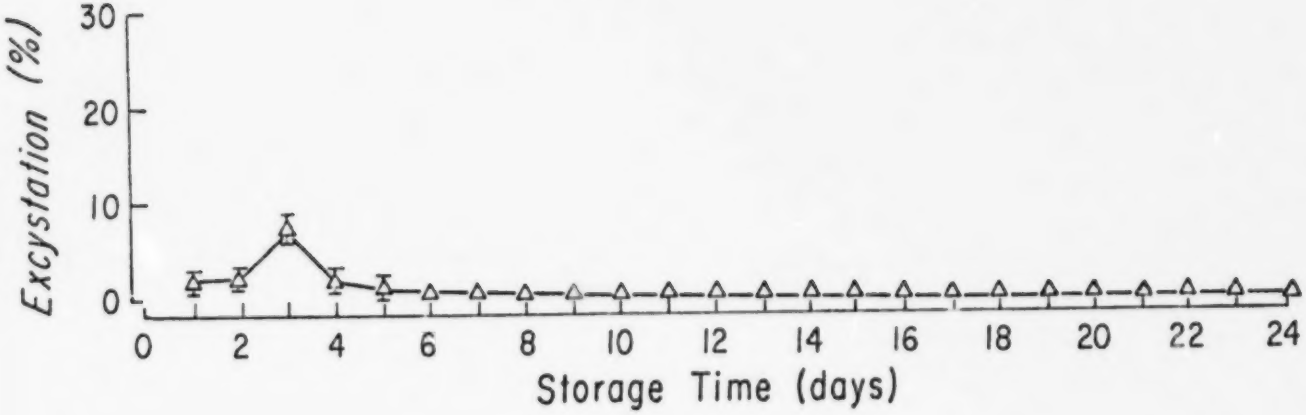

Figure 7. Effect of storage at 37°C on Giardia cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

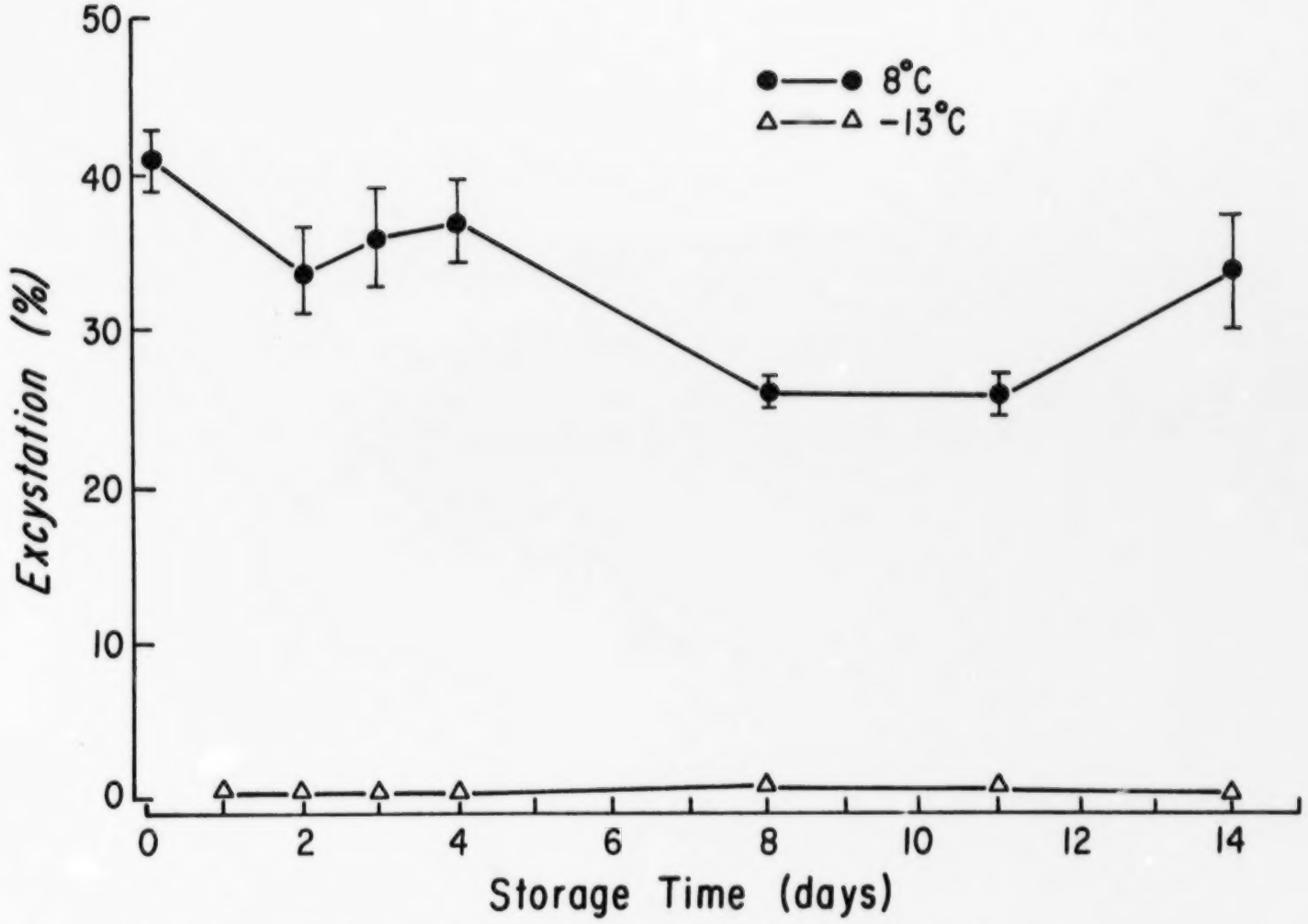

Figure 8. Effect of storage at -13°C on Giardia cyst viability as determined by excystation. Vertical bars represent standard error of the mean.

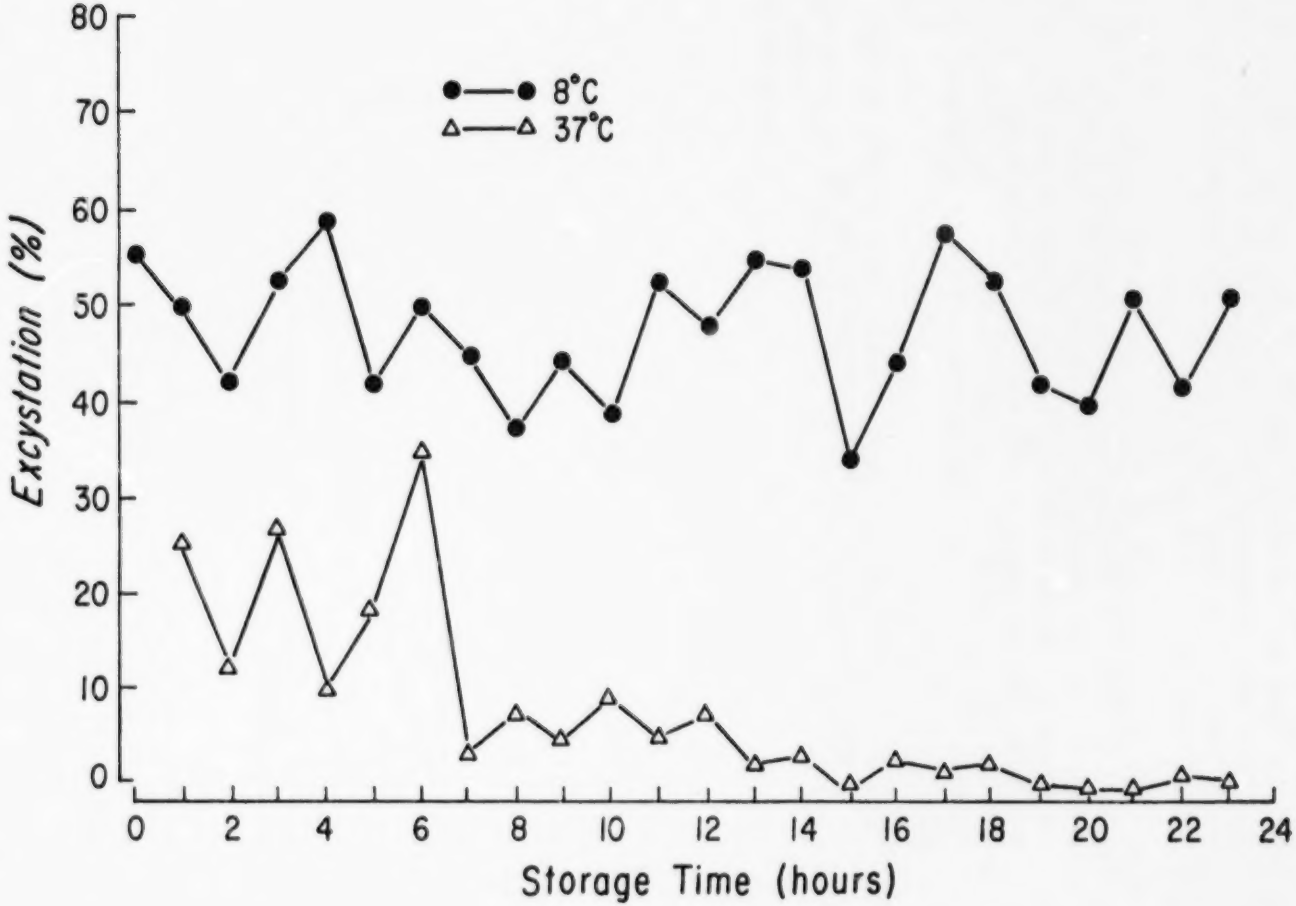

Figure 9. Effect of storage at 37°C on Giardia cyst viability as determined by excystation over a 24-hour period.

levels of excystation occurred when the temperature of both solutions was 37°C. Lower levels of excystation occurred with other temperature combinations. The percentage of excystation increased as the temperature of the acid, the HSP-3, or both was increased; the exception was that no excystation was induced when the temperature of the HSP-3 was held at 8°C, regardless of the temperature of the HCl. No change in the excystation percentages was noted after 18 hours incubation in HSP-3.

D. Post-acid incubation medium. The effect of the composition of the post-acid incubation medium on excystation was investigated by exposing cysts to HCl at pH 2.0 by the usual procedure, followed by their suspension in solutions of varying complexity and pH. Three excystation trials were performed counting an average total of 834 cysts in each solution. Significant levels of excystation were induced only in HSP-3 at pH 6.2 and 6.8 (Table 7). Further, excystation was significantly greater in medium at pH 6.8 than at pH 6.2 ($p<0.05$).

TABLE 7. EXCYSTATION OF GIARDIA EXPOSED TO HCl (pH 2.0) AND TRANSFERRED INTO SOLUTIONS OF VARYING COMPLEXITY AND pH

Post-acid Solution	pH	Percent Excystation *
Water	7.0	0.3
HCl	2.0	0.8
Saline (0.85 M)	7.0	0
PBS	7.0	1.1
HSP-3	0.5	0
	2.0	0
	4.0	0
	6.2	63.3
	6.8	100.0

* Percentages of excystation with reference to the control tube (pH 6.8 HSP-3) which is arbitrarily 100 percent.

The experiment was repeated as before, except that cysts were transferred into either 1) Hanks-phytone, 2) Hanks-phytone with serum, or 3) HSP-3 (Hanks-phytone with serum and NCTC-135), all at pH 7.0. Three trials were performed in which an average total of 1205 cysts were counted in each medium. It was found (Table 8) that the levels of excystation in HSP-3 and Hanks-phytone with serum, while not significantly different from each other ($p>0.05$), were significantly greater than excystation in Hanks-phytone alone ($p<0.05$).

TABLE 8. EXCYSTATION OF GIARDIA EXPOSED TO HCl (pH 2.0) AND TRANSFERRED INTO COMPONENT-VARIED HSP-3 AT pH 7.0

Solution	Mean * percent excystation ± S.E.M.
Hanks-phytone	13.1 ± 1.4
Hanks-phytone + serum	42.7 ± 3.1
HSP-3	50.9 ± 4.0

* Means are derived from an average total of 1205 cysts counted in each solution.

IV. Cyst variation.

A. Maturation, daily variation and viability. Throughout this study, inconsistent results were sometimes found when a single purified cyst preparation was used over a period of days or weeks. These results appeared to be the result of cyst variability due to aging. To study this phenomenon, purified cyst suspensions were prepared from different fecal specimens from the same host, stored at 8°C, and excystation in pH 2.0 HCl followed in each suspension over a period of weeks. Figure 10 is the excystation pattern for a single suspension of purified cysts over 11 weeks, and is representative of patterns obtained with other samples. Three excystation trials were performed daily; an average total of 451 cysts were counted each day. This pattern has three important features. First, as noted previously, cyst storage began with a four day "lag" period of low excystation, after which an increase in excystation percentages was seen. Secondly, following the lag period, high levels of excystation were seen until about day 30 during which period extreme fluctuations in excystation occurred almost daily. Thirdly, following the period of elevated excystation, these levels decreased rapidly for approximately a week, and then gradually diminished during the next six weeks, indicating a loss of viability. Although the specific pattern varied greatly, the following characteristics were observed in most suspensions with low-temperature storage. A lag period of one to seven days, followed by an increase in excystation over three days, preceded a one to four week period of elevated but fluctuating excystation; this was followed by a drop in excystation and an extended period (usually several weeks) of gradually diminishing low levels of excystation.

B. Variation in sensitivity to physical environment. Cysts were examined to determine if sensitivity to the physical environment changes during aging. Purified cysts stored for 47 days at 8°C were excysted periodically using pH 2.0 HCl at various exposure times up to 120 minutes. Three trials were performed counting an average total of 822 cysts at each temperature for each day tested. The results are summarized in Table 9. As the cysts aged, the mean optimum acid exposure time increased from 20 to 70 minutes. From days 34 to 47 the mean optimum time remained at 70 minutes,

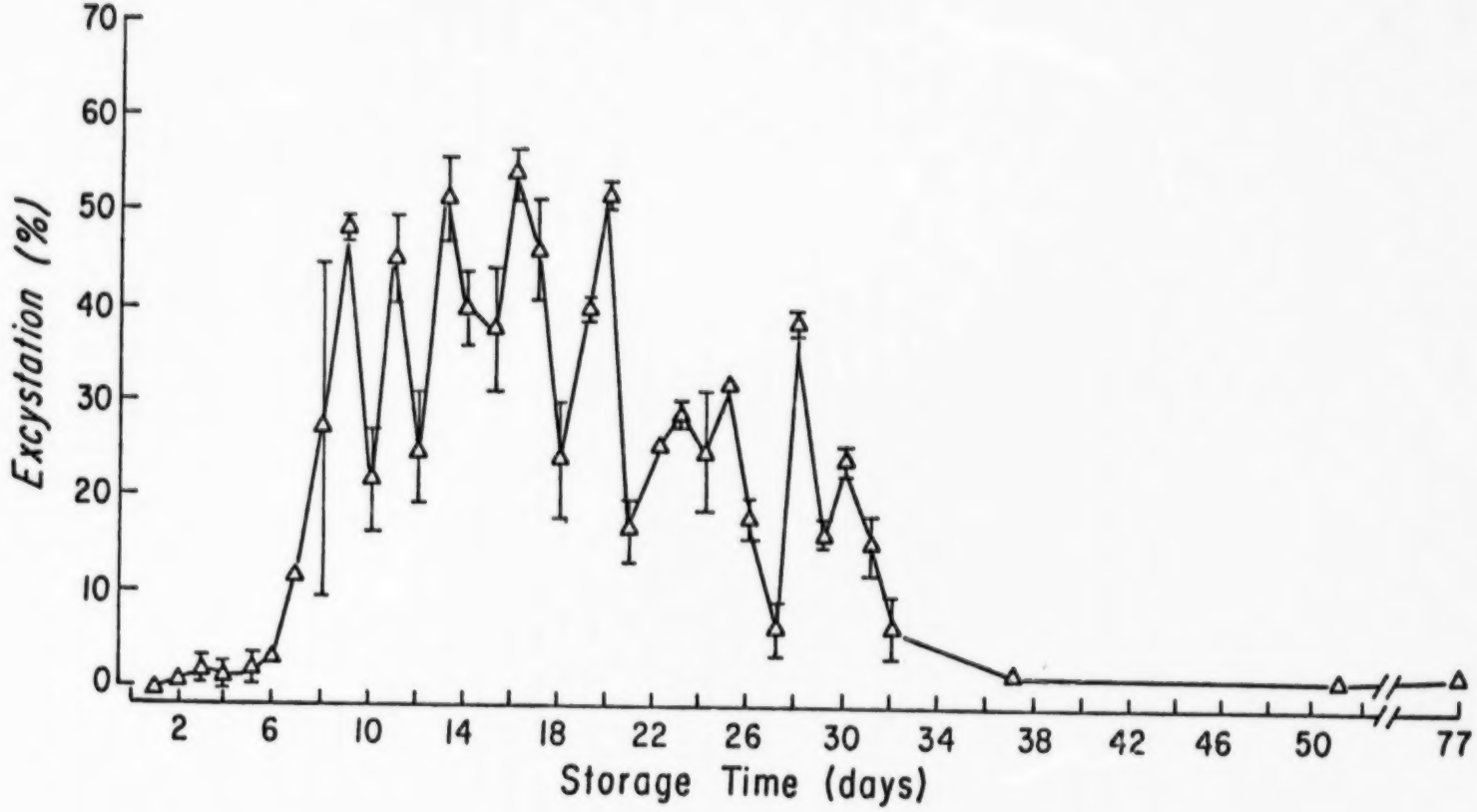

Figure 10. Giardia excystation pattern for cysts stored at 8°C for an 11-week period.

TABLE 9. OPTIMUM TIME OF ACID EXPOSURE FOR GIARDIA EXCYSTATION WITH AGING OF CYSTS

Day	Mean optimum exposure time * (min)	Range (min)	Mean optimum percent excystation ± S.E.M.#
1	20	20	18.1 ± 0.0
5	20	20	20.0 ± 0.0
9	35	10 - 60	8.5 ± 0.6
13	20	10 - 30	11.4 ± 0.4
16	20	0 - 30	8.4 ± 0.7
19	35	10 - 60	7.4 ± 0.4
23	40	20 - 60	6.5 ± 0.6
34	70	20 - 120	3.0 ± 0.4
47	70	20 - 120	1.9 ± 0.2

* Average of exposure times at which optimum percentages of excystation were not significantly different from each other on a particular day ($p>0.05$).

\# Average of optimum percentages of excystation which are not significantly different from each other on a particular day ($p>0.05$).

although it may have proved longer if an acid exposure time of more than 120 minutes had been tried. The minimal optimum exposure time (see Range, Table 9) did not change much during the storage period but remained between 10 and 20 minutes, whereas the maximal optimum exposure time increased from 20 to 120 minutes, resulting in the increasing mean optimum exposure time. Additionally, the mean optimum percentage of excystation showed a similar pattern as observed in previous storage experiments.

V. Culturing.

Using the procedure described previously with pH 2.0 HCl, excystation was induced in Giardia obtained from infected humans, monkeys, dogs, beavers, rats and mice, and cultures were established from excysted trophozoites of cysts from humans and monkeys. These cultures were maintained for up to seven months in HSP-3, and most were cultivated axenically from the time of excystation.

VI. Other reports based on this research.

Additional reports, based on the research conducted under this grant, include the following:

Bingham, A.K. and E.A. Meyer. The in vitro excystation of Giardia. Fourth International Congress of Parasitology (abstract), pp. 95-96, 1978.

Jarroll, E.L., Jr., A.K. Bingham, E.A. Meyer and S. Radulescu. Effect of temperature on Giardia cyst survival as determined by eosin staining and excystation. Fourth International Congress of Parasitology (abstract), p. 96, 1978.

Bingham, A.K., E.L. Jarroll, Jr., S. Radulescu and E.A. Meyer. Induction of Giardia excystation and the effect of temperature on cyst viability as compared by eosin-exclusion and in vitro excystation. Symposium: Waterborne transmission of giardiasis (in press), (U.S. Environmental Protection Agency, 1979).

Bingham, A.K. and E.A. Meyer. Giardia excystation can be induced in vitro in acidic solutions. Nature, 277:301-302, 1979.

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